Method For Inhibiting Pulmonary Oxygen Toxicity Using Manganese Superoxide Dismutase

Field of the Invention

This invention relates to a method for inhibiting pulmonary oxygen toxicity in a patient requiring elevated levels of oxygen, using human manganese superoxide dismutase. More particularly, this invention relates to a method for inhibiting pulmonary oxygen toxicity in a patient requiring elevated levels of oxygen, by prophylactic topical administration of human manganese superoxide dismutase in tetrameric form.

Background of the Invention

Bypass surgery, trauma, emboli, sepsis, pneumonia, smoke inhalation, radiation and chemotherapy, and premature birth frequently initiate acute lung injury, edema, and inflammation that result in impaired alveolar gas exchange. In such patients, the use of elevated levels of inhaled oxygen are required to achieve acceptable blood oxygen saturation. However, prolonged exposure to high concentration of oxygen can precipitate acute edematous lung injury (alveolar septal thickening), followed by fibrosis, pulmonary hypertension and/or bronchopulmonary dysplasia. In the presence of acute lung injury, that is in cases where elevated levels of oxygen may be required, normobaric oxygen at concentrations as low as 50% have been shown to potentiate the injury. Accordingly, agents that attenuate or delay the toxic effects of inhaled oxygen would be desirable.

The initiation of pulmonary oxygen toxicity seems to occur through a direct increase in the intracellular production of partially reduced, reactive oxygen species (superoxide, O_2; hydrogen peroxide, H_2O_2; and hydroxyl radical, OH•) that overwhelm intracellular anti-oxidant defense mechanisms inducing cell damage or death. Once these effects are initiated, the lung injury is amplified and morphologic changes become pronounced, exponentially between 48 and 72 hours of hyperoxia presumably due to the

extracellular release of cytotoxic enzymes and reactive oxygen metabolites arising from a notable neutrophil margination and infiltration. These same processes often also underlie the development of the acute lung injuries that make hyperoxia necessary.

The role of intracellular reactive oxygen species has been underscored by the "tolerance" to pulmonary oxygen toxicity achieved via augmenting intracellular anti-oxidants through pretreatment of animals with non-lethal exposures to endotoxin, ozone, bleomycin, or hyperoxia. The specific contribution of superoxide dismutase (SOD) to intracellular anti-oxidant defenses and hyperoxia resistance has been shown by the marked protection produced after the selective up-regulation of mitochondrial manganese superoxide dismutase (MnSOD) expression following tumor necrosis factor (TNF) pretreatment or hypoxic exposure in rats as well as by the transgenic enhancement of cellular CuZnSOD or MnSOD in mice. However, the exogenous addition of SOD alone (i.e., without catalase) has so far proven ineffective or only very weakly effective in preventing the severe lung injury induced by continuous pure oxygen breathing.

European Patent Application No. 0,284,105 describes, inter alia, the use of human MnSOD to treat lung fibrosis by intravenous, intratracheal or, preferably, subcutaneous administration. This application describes the enzymatically active form of human MnSOD as a protein having at least two, and possibly four, identical subunits.

U.S. Patent No. 5,116, 616 describes a method of protecting a human from lung injury due to hyperoxia and hyperventilation by the intratracheal administration of free CuZnSOD. This patent also describes substituting MnSOD for the CuZnSOD in this method, the MnSOD being of human, bovine or other mammalian origin.

Prior to the present invention, it was not known that the tetrameric form of human MnSOD possessed unexpectedly superior ability to ameliorate the effects of pulmonary oxygen toxicity when administered topically.

Accordingly, it is the purpose of this invention to provide an improved method for preventing pulmonary oxygen toxicity by the prophylactic topical administration of a human MnSOD tetramer.

Summary of the Invention

The present invention relates to a method for inhibiting pulmonary oxygen toxicity in a patient susceptible thereto, which comprises topically and prophylactically administering to the patient, an effective amount of a human MnSOD tetramer.

Brief Description of the Figures

Figure 1A is a graphic depiction of SOD activity in whole lung lavage after intranasal insufflation (inhalation) administration of MnSOD or CuZnSOD. Values are mean + S.E. ($n \geq 4$ per time point).

Figure 1B is a graphic depiction of SOD activity in lung homogenate after intranasal insufflation (inhalation) administration of MnSOD or CuZnSOD. Values are mean + S.E. ($n \geq 4$ per time point).

Figure 2 is a graphic depiction of the effects of intranasal (inhalation) administration of MnSOD or CuZnSOD compared to vehicle (saline) on the decrease in Crs and increase in lung lavage LDH activity induced by exposure to pure oxygen for 84 hours. Values are mean + S.E. Single or double asterisks signify significant protection versus vehicle (saline) or both vehicle and CuZnSOD treatment(s), respectively ($p < 0.05$, Dunnett's t-test).

Figure 3A is a graphic depiction of the effects of intranasal insufflation (inhalation) administration of MnSOD [active (+) and heat inactivated (-)] or CuZnSOD, compared to vehicle (saline), on the decreases in Crs and D_{LCO} induced by exposure to pure oxygen for 84 hours. Values are mean + S.E.

Single or double asterisks signify significant protection versus just vehicle or both vehicle and inactivated MnSOD treatment(s), respectively ($p < 0.05$, Dunnett's t-test).

Figure 3B is a graphic depiction of the effects of intranasal insufflation (inhalation) administration of MnSOD [active (+) and heat inactivated (-)] or CuZnSOD, compared to vehicle (saline), on the increases in lung lavage LDH activity and total protein content induced by exposure to pure oxygen for 84 hours. Values are mean + S.E. Single or double asterisks signify significant protection versus just vehicle or both vehicle and inactivated MnSOD treatment(s), respectively ($p < 0.05$, Dunnett's t-test).

Figure 4A is a graphic depiction of the effects of intranasal insufflation (inhalation) administration of MnSOD compared to vehicle (saline) on the decreases in Crs and D_{LCO} produced by the enhancement of a BHT (300 mg/kg, i.p.) induced acute lung injury by hyperoxia (6 days of 65% oxygen exposure) as measured on day 14. Values are mean + S.E. Asterisks signify significant protection versus vehicle treatment ($p < 0.05$, Dunnett's t-test).

Figure 4B is a graphic depiction of the effects of intranasal insufflation (inhalation) administration of MnSOD compared to vehicle (saline), on the increase in lung collagen and decrease in body weight produced by the enhancement of a BHT (300 mg/kg, i.p.) induced acute lung injury by hyperoxia (6 days of 65% oxygen exposure) as measured on day 14. Values are mean + S.E. Asterisks signify significant protection versus vehicle treatment ($p < 0.05$, Dunnett's t-test).

Figure 5 is a series of photographs showing representative 5μm lung sections stained with Masson's Trichrome from naive animals (A and E) as well as from animals with lung inflammation and fibrosis (blue staining) produced by BHT plus hyperoxia and treated with vehicle (saline) i.p. or i.n. (B and F), MnSOD at 10 mg/kg, i.p. (C and G), or MnSOD at 1 mg/kg, i.n. (D and H). Magnification is 10x for A-D and 40x for E-H. Topical administration of MnSOD provided protection whereas systemic administration of MnSOD did not.

Figure 6 is a graphic depiction of the effects of intranasal insufflation (inhalation) administration of MnSOD, heat inactivated MnSOD, or CuZnSOD compared to vehicle (saline), on survival after the enhancement of a BHT (300 mg/kg, i.p.) induced lung injury by hyperoxia (6 days of 65% oxygen exposure) (n=10/group).

Figure 7 demonstrates protein-A gold immunolabeling (black dots) for hrMnSOD 4 hours after insufflation into the lungs. Note that hrMnSOD is highly retained on alveolar surface (A). No labeling is present on the capillary surface (C). The lungs were fixed by vascular perfusion.

Figure 8 demonstrates immunohistochemical staining (red) for hrMnSOD after intranasal insufflation (A) versus aerosol inhalation (B and C). Magnification is 4x in A and B, and 10x in C. Note the nonuniform delivery in A versus uniform in B. Also note that the hrMnSOD is located almost exclusively in the fluid lining the airspaces.

Figure 9 shows representative electron microscopy of the alveolar septa: (A) from a naive (room air) control mouse demonstrating the normal thin air-blood barrier (arrow). (B) from a mouse exposed to pure oxygen for 84 hours and treated only with aerosolized phosphate buffered saline (vehicle for hrMnSOD) for 1 hour/day. Note the destruction of all major tissue compartments plus a neutrophil (N) infiltrate. The epithelium is extensively damaged or gone (short arrows), endothelium (E) is damaged (open arrow), and there is marked interstitial edema (*). (C) from a mouse exposed to pure oxygen for 84 hours and treated for 1 hour/day with aerosolized rhMnSOD (1 mg/ml). Note only minimal tissue injury compared to the vehicle treated hyperoxic injury. Areas of endothelial swelling (short arrow) and of mitochrondial swelling (long arrow) are shown.

Figure 10 demonstrates arterial blood oxygen content (partial pressure; PaO_2 in mmHg) over time (hours) in adult baboons during anesthesia, mechanical ventilation and continuous exposure to pure oxygen. The mean ± standard error for four animals treated with aerosolized hrMnSOD

(3 mg/kg/day, q 12 hours) (top line; solid circles) compared to seven historical control animals (bottom line; open squares) are plotted. Differences (protection) are highly significant ($p < 0.05$) by ANOYA.

Figure 11 shows representative electron microscopy of the alveolar septa: (A) from a control baboon. (B) from a baboon exposed to pure oxygen for 96 hours. Note diffuse epithelial destruction (long arrows), endothelial cell damage (short arrows) and interstitial edema (*). (C) from a baboon treated with aerosolized rhMnSOD (3 mg/kg/day, q 12 hours) while begin exposed to pure oxygen for 96 hours. rhMnSOD gave obvious protection against the diffuse septal destruction caused by hyperoxia.

Detailed Description of the Preferred Embodiments

This invention relates to an improved method for inhibiting pulmonary oxygen toxicity in a patient susceptible thereto which comprises prophylactically administering to the patient, by topical administration, an effective amount of a human MnSOD tetramer.

Pulmonary oxygen toxicity for the purposes of this invention is defined as the acute lung injury (dysfunction) and chronic lung scarring (fibrosis or bronchopulmonary dysplasia), or death due to lung dysfunction, caused or enhanced by oxygen inhalation. Pulmonary oxygen toxicity results from inhalation of elevated levels of oxygen, such as, for example, greater than 0.5 atm for greater than 24 hours.

A human MnSOD tetramer for the purposes of this invention is defined as a polypeptide with four identical subunits wherein the four identical subunits have the same or substantially the same, amino sequence as the four subunits of naturally occurring human MnSOD tetramer and wherein the polypeptide has the same or substantially the same, biological activity as naturally occurring human MnSOD.

A human MnSOD tetramer useful in the method of this invention can be prepared as described in U.S. Patent No. 5,260,204 or EP 282,899.

Preferably, the human MnSOD tetramer useful in the method of this invention is prepared by transforming a eukaryotic host with a vector comprising the sequence

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5'    ATG AAG CAC TCT TTG CCA GAC TTG CCA TAC GAC TAC GGT
      GCT CTA GAA CCA CAC ATC AAT GCT CAA ATC ATG CAA TTG
      CAC CAC TCT AAG CAC CAC GCG GCC TAC GTG AAC AAC CTG
      AAC GTC ACC GAG GAG AAG TAC CAG CAG GCG TTG GCC AAG
      GGA GAT GTT ACA GCC CAG ATA GCT CTT CAG CCT GCA CTG
      AAG TTC AAT GGT GGT GGT CAT ATC AAT CAT AGC ATT TTC
      TGG ACA AAC CTC AGC CCT AAC GGT GGT GGA GAA CCC AAA
      GGG GAG TTG CTG GAA GCC ATC AAA CGT GAC TTT GGT TCC
      TTT GAC AAG TTT AAG GAG AAG CTG ACG GCT GCA TCT GTT
      GGT GTC CAA GGC TCA GGT TGG GGT TGG CTT GTT TTC AAT
      CAG GAT CCA CTG CAA GGA ACA ACA GGC CTT ATT CCA CTG
      CTG GGG ATT GAT GTG TGG GAG CAC GCT TAC TAC CTT CAG
      TAT AAA AAT GTC AGG CCT GAT TAT CTA AAA GCT ATT TGG
      AAT GTA ATC AAC TGG GAG AAT GTA ACT GAA AGA TAC ATG
      GCT TGC AAA AAG TAA      [SEQ ID NO:1]
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wherein AAG at codon 30 can be replaced by CAG and CAC at codon 32 can be replaced by CAT [SEQ ID NO:2], [SEQ ID NO:3] and [SEQ ID NO:4]. Preferably, the eukaryotic host is yeast.

Methods for the topical administration of the human MnSOD tetramer, for the purposes of this invention, include intranasal insufflation, inhalation and intratracheal instillation (with or without the co-administration of surface tension lowering agents to enhance spreading).

In providing a patient with the human MnSOD tetramer useful in the method of this invention, the dosage of administered human MnSOD tetramer will vary depending upon such factors as the patient's age, size, lung volume, health, duration of exposure, barometric pressure of the inhaled gas mixture, composition of inhaled gasses, P_aO_2, etc. In general, it is desirable to provide the patient with a dosage of administered agent in the range of from about 0.01-10.0 mg/kg (body weight of patient), although a lower or higher dosage may be administered.

The human MnSOD tetramer useful in this invention, may be administered prophylactically, i.e., in advance of the onset of pulmonary oxygen toxicity. The prophylactic administration of the human MnSOD tetramer serves to inhibit or attenuate any subsequent toxic response to the oxygen.

The human MnSOD tetramer can be administered in a single prophylactic dose or in multiple prophylactic doses. Preferably, the multiple doses are administered at regular intervals during the period of time that the patient is exposed to the elevated level of inhaled oxygen.

The human MnSOD tetramer useful in the method of this invention can be formulated according to known methods to prepare pharmaceutically useful composition, such as, for example, whereby the agent is combined in admixture with a pharmaceutically acceptable carrier, including a surface tension lowering agent, such as a surfactant, to enhance spreading distribution. Suitable vehicles and their formulations are described, for example, in *Remington's Pharmaceutical Sciences* (16th ed., Osol, A., Ed., Mack, Easton PA (1980)).

The following examples are an illustration of the present invention.

Examples

Preparation of MnSOD

Human MnSOD tetramer was prepared as described in U.S. Patent No. 5,260,204 and EP 282,899. In brief, MnSOD was produced in the yeast *Saccharomyces cervisiae* using the human MnSOD gene linked to a mitochondrial leader sequence as the expression plasmid. The leader peptide was removed during the passage of the preprotein present in crude extracts. The MnSOD was purified, sterile filtered, stored at protein concentrations of 1.7 - 5.0 mg/ml at 4°C in 20 mM phosphate buffer with 150 mM NaCl (pH 7.2), and had specific activities of 3100 - 3580 U/mg, as determined by the cytochrome C assay described in McCord and Fridovich, *J. Biol. Chem. 244*:6049 (1969), and endotoxin < 2.70 EU/mg.

Bovine CuZnSOD with a specific activity of 3250 U/mg was obtained commercially (Cat. No. S-2515, Sigma Chemical Co., St. Louis, MO). Endotoxin levels were determined to be < 2 EU/mg.

As a negative control, human MnSOD tetramer as prepared above, was heat inactivated by boiling for 30 minutes. The specific activity of the heat inactivated MnSOD was < 5 U/mg, as determined by a modified form of the cytochrome C assay described in McCord and Fridovich, *supra*. (see Example 1).

Example 1

Male Balb-c mice (Charles River, Raleigh, NC) weighing 21 to 25 g. were used to determine the enhancement of lung SOD activity at various time points after dosing with MnSOD or CuZnSOD. Topical administration was performed using a 100 μl pipetter to apply a total of 50 μl to the nostril openings of mice lightly anesthetized with inhaled methoxyflurane (Metafane, cat NDC 11716-5493-4; Pitman-Moore, Inc., Mundelein, IL) and held in a gravity favoring vertical orientation.

At various time points thereafter, the mice were anesthetized with 60-90 mg/kg, i.p. sodium pentobarbital (Nembutal, cat NDC 0074-3778-05; Abbott Labs, North Chicago, IL) and a tracheal cannula (Teflon catheter cut to 2 cm with Luer Plug, 18 gauge, cat 2N112; Baxter Healthcare Corp., Deerfield, IL) inserted. A ventral midline incision was made in the upper abdomen, the diaphragm ruptured, the chest plate removed and the left ventricle cut inducing exsanguination into the thoracic cavity. Whole lung lavage was then performed via the tracheal cannula by twice gently injecting and then withdrawing 1 ml aliquots of phosphate (0.05mM, pH 7.4) buffered saline (PBS). The aliquots were combined, the cells pelleted by centrifugation at room temperature and 350 x g for 10 min., and the supernatants decanted and stored in cryovials at -20°C until assayed. The lung vasculature was then rid of any residual blood using a retrograde perfusion with room temperature PBS. The lungs were excised and the lobes minus all large airways and vessels placed into a high speed polycarbonate centrifuge tube containing 1 ml of 2% Triton-X in 0.1M phosphate buffer, pH 7.8., The lobes were then

homogenized (Kinematica: Polytron Tissue Homogenizer, model PT 10-35; Brinkman Instruments, Westbury, NY), the homogenate centrifuged at 4°C and 20,000 x g for 20 min., and the supernatant decanted and stored in cryovials at -20°C until assayed.

For the measurement of lung lavage and homogenate SOD activity, the xanthine-xanthine oxidase induced reduction of cytochrome C assay described by McCord and Fridovich, *supra*, was modified to a 96-well microtiter plate format. In brief, 10 μl of sample plus 10 μl of 0.1 U/ml xanthine oxidase (cat. #4500; Sigma Chemical Co., St. Louis, MO) or distilled water (as a blank) were added to triplicate wells. Optical density (OD) at 415 nm was then recorded (model 340 ATCC; SLT Lab instruments, Hillsborough, NC) immediately and 7 min. after adding 150 μl of the reaction mixture containing 2 x 10^{-4}M EDTA, 1 x 10^{-4}M xanthine (cat. #X-0250; Sigma Chemical Co.), 2 x 10^{-5}M KCN, and 3 x 10^{-5}M cytochrome C (cat. #C-2506; Sigma Chemical Co.) in 0.1M potassium phosphate buffer (pH 7.8). The mean change in OD for each sample/dilution minus its corresponding blank was then compared to a standard curve constructed with stock MnSOD. The specific activity of the stock MnSOD was determined in the standard McCord and Fridovich assay.

Results

Intranasal administration of MnSOD at 10 mg/kg caused huge (peak > 100-fold) and prolonged (still 25-fold at 48 hours) increases in lung lavage SOD activity (Figure 1A). At 1 and 0.1 mg/kg more moderate (peaks of 10-fold and 3-fold, respectively) but similarly prolonged rises in lavage SOD activity were found. Lung homogenate SOD activity was elevated by approximately 100% for 24 hours by MnSOD at 10 mg/kg and only slightly by the 1 and 0.1 mg/kg doses (Figure 1B). Intranasal CuZnSOD at 10 mg/kg produced an augmentation of lung lavage and homogenate SOD activity that was similar in magnitude and duration to that found for MnSOD (Figures 1A

and 1B). Based on these results, MnSOD at doses of 10, 1 and 0.1 mg/kg, intranasal (i.n.) every 24 hours (s.i.d.) were compared to CuZnSOD at 10 and 1 mg/kg, i.n., s.i.d., in two murine models of pulmonary oxygen toxicity described below in Examples 2 and 3.

Example 2

Male Balb-c mice (Charles River, Raleigh, NC) weighing 23 to 26 g were housed on wire mesh above bedding in a clear plexiglass chamber (71 x 54 x 33 cm; 40-60 mice/experiment; 8-12 mice/treatment group) and exposed to 100% oxygen at atmospheric pressure for 84 hours. Food (laboratory mouse chow) and water were supplied *ad libitum* and the chambers cleaned once every 24 hours. Chamber oxygen, humidity and temperature were monitored continually using a digital oxygen monitor (Gas Tech. Inc., model 6873; from Lab Safety Supply, Janesville, WI) and wall-mount hygrometer/thermometer (Oakton, model 3313-90; Cole Palmer Instrument Co., Chicago, IL), respectively. Chamber air was circulated by a three-inch fan and carbon dioxide accumulation was eliminated by mixing soda lime (cat. S-200, 4-8 mesh, indicator grade; Fisher Scientific, Springfield, NJ) in the bedding. During these experiments, chamber oxygen was $\geq$ 97%, humidity ranged from 65-80% and temperature from 22.5-24.5°C. MnSOD or CuZnSOD was administered daily by intranasal insufflation (inhalation; as described above in Example 1) staring just before the onset of hyperoxic exposure and continued to the end of the experiment.

At the end of each protocol, the mice were anesthetized with 60-90 mg/kg, i.p., sodium pentobarbital and a tracheal cannula (as described above in Example 1) inserted. Three tidal volume (1.2-1.5 ml) breaths were given by connecting a syringe to the tracheal cannula to eliminate areas of atelectasis and provide a constant volume history for the lung function measurements described below.

Respiratory system impedance (Zrs) was measured by discrete frequency (4 to 40 Hz in 11 equal logarithmic steps) sinusoidal forced oscillations (amplitude of 1 ml/sec ± 20% at each frequency) superimposed on tidal breathing using a technique as described in Dorkin *et al., J. Appl. Physiol.: Respirat. Environ. Exercise Physiol. 52*:1097 (1982) and Jackson *et al., Respir. Physiol. 48*:309 (1982). Advantages of this technique include that all transducers used are frequency response corrected, the impedance of the tracheal cannula is accurately eliminated, and the measurements are independent of the ventilatory pattern (tidal volume and respiratory rate) of the animal. Respiratory system compliance (Crs) was computed from Zrs as described in Jackson *et al., supra*.

Diffusion capacity of the lungs (D_{LCO}) was measured by the carbon monoxide single breath method as described in Forster *et al., J. Clin. Invest. 33*:1135 (1954). In brief, 1.0 ml of gas containing 0.479% carbon monoxide, 0.477% neon, 20.22% oxygen and 78.824% nitrogen (cat. NDC 11939-3935; Metheson Gas Products, Inc., East Rutherford, NJ) was injected into the lungs through the tracheal cannula. Ten seconds later, a 0.5 ml dead space/airway sample was withdrawn followed immediately by the withdrawing of the 0.5 ml alveolar sample. The alveolar gas sample was analyzed using a gas chromatograph (model 01111, Carle AGC series 100; Carle Chromatography, Loveland, CO). The values for D_{LCO} obtained for the unexposed mice (see Results section below) are similar to those described for CBA/Ca Lac Cbi mice using a rebreathing method in Depledge *et al., Int. J. Radiation Oncology Biol. Phys. 7*:485 (1981).

After the measurement of lung function, whole lung lavage was performed as described above in Example 1, to assess leukocyte infiltration, cell damage, and lung microvascular fluid leak. Total leukocytes per ml of lavage was determined with a Coulter counter (model ZM; Coulter Electronics, Hialeah, FL). Differential cell counts (200 cells counted) were performed on Wright-Giemsa-stained cytocentrifuge (Cytospin, model 2; Shandon, Oakland, CA) preparations. The lung lavage fluid then was

centrifuged at 600 x g for 10 minutes to remove cells and particulates, after which the supernatant was collected and aliquoted: 0.5 ml kept at 4°C for LDH activity measurement (within 24 hours) and the remaining portion frozen at -70°C for the measurement to total protein. Lactate dehydrogenase (LDH) activity was measured using a commercially available kit (cat. 44564; Roche Diagnostic Systems, Inc., Nutley, NJ). Total protein in the lung lavage supernatant was measured by a modified Lowry method also using a commercially available kit (BCA Protein Assay Reagent 23225; Pierce, Rockford, IL).

Results

In two separate experiments, MnSOD was found to provide a dose dependent inhibition of the decreases in Crs and D_{LCO} (65% and 42%, respectively, at 1 mg/kg, optimal dose) and increase in lung lavage protein (82% at 1 mg/kg) as well as a dose independent inhibition of the increase in lung LDH activity (~58%) induced by 84 hours of pure oxygen exposure (Figures 2, 3A and 3B). CuZnSOD at 10 mg/kg and heat inactivated MnSOD at 1 mg/kg were ineffective while CuZnSOD at 1 mg/kg conferred only a weak inhibition of the decrease in Crs (43%), having no effect on the decrease in D_{LCO} or the increase in lung lavage protein and LDH (Figures 3A and 3B).

Example 3

Male Balb-c mice (Charles River, Raleigh, NC) (40-60 mice/experiment; 8-12 mice/treatment group) weighing 24 to 27 g were injected with butylated hydroxytoluene (BHT, 300 mg/kg, i.p. in corn oil 4 mg/kg) to induce an acute lung injury followed 2 hours later by 65% oxygen for 6 days. The site and type of the injury induced by BHT, necrosis of alveolar type I epithelial cells, is probably the most common form leading to the onset of ARDS. The hyperoxic exposure (performed as described in

Example 2 above; actual chamber oxygen ranged from 63-67%) enhanced this lung injury, as well as delaying and/or preventing normal repair, resulting in lung scarring (fibrosis) at day 14.

At day 14, pulmonary function and inflammation were assessed as described above in Example 2. The lungs were the excised with the tracheal cannula still in place. The right main stem bronchus was occluded just below the carina via suture and the right lung lobes removed, homogenized (see Example 1 above), frozen (-70°C for ≥ 16 hours), and then hydrolyzed in 12N HCl for 18 hours at 110°C. Hydroxyproline content (lung collagen/fibrosis) was then determined by a standard colorimetric assay as described in Stegemann *et al., Clin. Chim. Acta 18*:267 (1967). The left lung was then filled via the tracheal cannula with 10% neutral buffered formalin (NBF), occluded via suture, placed into a plastic specimen cassette, and stored submerged in 10% NBF. For examination by light microscopy, the fixed lungs were embedded in paraffin, microtomed into 5 μm sections, and stained with hematoxylin and eosin for histopathologic scoring or with Masson's Trichrome for collagen (fibrosis) scoring. A qualitative - to ++++ scoring was performed by an individual blind to the protocol and treatment. Whole lung lavage was not performed on the animals used for histology.

Results

Intranasal administration of MnSOD provided impressive protection of the lung dysfunction, inflammation and fibrosis induced by BHT plus hyperoxia. The reduction in Crs and D_{LCO} were completely inhibited at 1 mg/kg (Figure 4A), while the increase in lung collagen (fibrosis) and decrease in body weight were inhibited by ≥ 65% at all doses tested (Figure 4B). Histologically, BHT plus hyperoxia induced a marked perivascular and peribronchial inflammation (increase in foamy macrophages, eosinophils, monocytes and neutrophils), intravascular neutrophil margination, necrosis (karyorrhexis) of alveolar type I epithelial cells, hyperplasia and hypertrophy

of alveolar type II epithelial cells, and alveolar septal thickening as well as fibrosis. Intranasal, but not intraperitoneal, administration of MnSOD strikingly inhibited these histologic alterations (Table I below and Figure 5).

TABLE I

Effects of Systemic and Topical MnSOD on the Histology Alternatives Produced by the Enhancement of BHT-Induced Acute Lung Injury by Hyperoxia

Treatment Group	Leukocyte Infiltrate	Type I Pneumocyte Necrosis	Type II Pneumocyte Proliferation	Alveolar Wall Thickening	Collagen (Masson's Trichrome)
Naive	1.1 ±0.2	0.1 ±0.1	0.1 ±0.1	0.0 ±0.0	0.0 ±0.0
Saline (ip)	3.5 ±0.5	1.5 ±0.5	2.5 ±0.5	2.5 ±0.5	2.0 ±1.0
MnSOD (0.1 mpk, ip)	2.9 ±0.4	2.1 ±0.3	2.9 ±0.4	2.9 ±0.4	2.1 ±0.4
MnSOD (1.0 mpk, ip)	3.2 ±0.3	2.2 ±0.3	3.0 ±0.2	3.0 ±0.2	1.9 ±0.4
MnSOD (10 mpk, ip)	3.0 ±0.4	2.0 ±0.4	2.6 ±0.4	2.7 ±0.4	1.9 ±0.4
Saline (in)	3.0 ±0.4	1.9 ±0.5	2.0 ±0.4	2.0 ±0.5	1.9 ±0.5
MnSOD (0.1 mpk, in)	0.9 ±0.3*	0.0 ±0.0*	0.0 ±0.0*	0.0 ±0.0*	0.0 ±0.0*
MnSOD (1.0 mpk, in)	1.0 ±0.0*	0.0 ±0.0*	0.0 ±0.0*	0.0 ±0.0*	0.0 ±0.0*
MnSOD (10 mpk, in)	1.3 ±0.2*	0.0 ±0.0*	0.4 ±0.2*	0.0 ±0.0*	0.0 ±0.0*

Values are mean ± S.E. of qualitative scoring [0 (low) to 4 (high)] of H&E and/or Masson's Trichrome stained lung sections performed by an individual blind to the protocol and treatments.

* $p < 0.05$ versus vehicle (saline) treated (n = 3 or 4 per group)

In an additional experiment using slightly younger mice (weighing 21-23 g), the enhancement of the BHT-induced acute lung injury by hyperoxia resulted in death of 60% of the vehicle treated animals. MnSOD (at 0.01 or 1 mg/kg), but not CuZnSOD (at 1 or 10 mg/kg), was completely protective (Figure 6).

Example 4

Immunocytochemistry at both the light (biotin-streptavidin-horseradish peroxidase) and electron microscopic levels (protein-A gold) were employed to examine the distribution and retention of hrMnSOD given to mice by intranasal insufflation.

Mice were given a single dose (10 or 20 mg/kg; as described in Example 1) of either hrMnSOD or bovine serum albumin (BSA) and killed 0, 4, 12 or 48 hours later. Their lungs were fixed by vascular perfusion using 2% paraformaldehyde + 0.2% glutaraldehyde. Tissue sections were processed for light microscopy (cryosections) and electron microscopy (LR White embedding). Sections were labeled with rabbit anti-hrMnSOD polyclonal antisera alone or double labeled with anti-hrMnSOD and anti-surfactant protein A (SPA) antisera.

Results

hrMnSOD was found to remain in the extracellular lining fluids, primarily on the alveolar epithelial surfaces (Fig. 7), that is, optimally positioned to protect the primarily site of lung injury in pulmonary oxygen toxicity (the alveolar epithelium). The intensity of labeling did not change appreciably over the 48 hours of the study consistent with the SOD activity results reported in Example 1, Fig. 1. Only small amounts of material (anti-hrMnSOD staining) was found to move into the intracellular clefts or interstitial spaces. Alveolar macrophages had heavy labeling for hrMnSOD in vesicles that also labeled for SPA.

Thus, intranasal administration of hrMnSOD distributes primarily throughout the epithelial surface lining fluid and has a significantly longer retention than the reported 6 hour half-life when given systemically. These

properties explain the protective effects of hrMnSOD in oxidant-mediated lung disease, such as pulmonary oxygen toxicity, which involve reactions on the epithelial surface, as demonstrated in Examples 2 and 3.

Example 5

Immunocytochemistry at the light microscopic level was used to elevate the uniformity of distribution of hrMnSOD in mice after intranasal insufflation and aerosol inhalation administration.

Biotin was linked to hrMnSOD by standard techniques. Mice were given a single dose by intranasal insufflation (10 mg/kg) or aerosol inhalation [mice were placed a sealed chamber (Rubbermaid 2.4 quart servin' saver; ~20 cm diameter x ~8 cm high) with the output of (aerosol generated by) a DeVilbiss Aerosonic ultrasonic nebulizer loaded with hrMnSOD at 10 mg/ml passed through the chamber at 1 liter/minute for 30 minutes] and killed 0 to 1 hour later. The lungs were fixed by vascular perfusion using 2% paraformaldehyde + 0.2% glutaraldehyde. Cryosections of lung tissue were then immunostained by adding streptavidin linked horseradish peroxidase followed by 3-amino-9-ethylcarbazole (AEC) as substrate and Mayer's hematoxylin as counterstain.

Results

Intranasal administration resulted in a nonuniform delivery of hrMnSOD (typically proximal to the hilum region on the right side) (Fig. 8A). In contrast, aerosol inhalation resulted in deposition of hrMnSOD into all regions of the lungs (Fig. 8B). Examination at high magnification showed that, where deposited, hrMnSOD remained primarily in the epithelial lining fluid (Fig. 8C) as found in Example 4 above.

Thus, aerosol inhalation in comparison to intranasal insufflation provides a more uniform/complete coverage of (and thus protective potential for) the lung gas exchange surface area.

Example 6

Aerosol administration of hrMnSOD (as described in Example 5) was evaluated for protection of pulmonary oxygen toxicity using the murine model of 84 hours of pure oxygen breathing (as described in Example 2).

Results

In three separate experiments, inhaled hrMnSOD was found to provide marked protection of hyperoxic-induced lung injury. In the first experiment, a dose dependent, and complete at the highest dose, inhibition of the hyperoxia-induced increase in lung stiffness (decrease in Crs), decrease in the ability of the lungs to exchange gases (decrease in D_{LCO}), increase in lung microvascular leak (increase in lung lavage protein), and lung cell injury (increase in lung lavage LDH) was noted (Table II). Histologic protection of the injury for the mid-dose is shown in Figure 9. In the second experiment, in which the hyperoxic injury was more severe, both doses of inhaled hrMnSOD provided significant protection of all parameters evaluated even though both doses seemed to induce a leukocyte infiltration (Table III). In the third experiment, once daily treatment with inhaled MnSOD was also found to provide dose dependent and nearly complete inhibition of the hyperoxic-induced lung dysfunction and injury (Table IV).

Thus, aerosol administration of hrMnSOD (unlike any previous report for CuZnSOD in adult mammals) provides marked prevention of hyperoxic-induced lung injury.

Example 7

Aerosol administration of hrMnSOD is presently being evaluated for protection of the hyperoxic lung injury induced by pure oxygen during mechanical ventilation for 96 hours of anesthesia and intubated adult baboons [methodology as described in Fracica *et al., Exp. Lung Res. 14*:869 (1988)].

Results

Preliminary studies (N = 4) indicate that hrMnSOD (3 mg/kg/day, q 12 hours) gives dramatic protection as measured by physiologic parameters during the course of the oxygen therapy. This is best illustrated by the arterial partial pressure for oxygen (PaO_2) as shown in Figure 10. Light and electron microscopic analysis of lung sections obtained at the end of the hyperoxic exposure (96 hours) also demonstrate substantial protection by hrMnSOD. Figure 11A is an electron micrograph of an alveolar septum from a baboon given sedation and mechanical ventilation but no injury or therapy. Sedation and mechanical ventilation do not cause injury. Following exposure to 100% O_2 for 96 hours, the baboon lung demonstrates diffuse alveolar damage. Figure 11B illustrates a typical alveolar septum with marked destruction of the alveolar epithelium, hyaline membranes and fibrin on the alveolar surfaces, interstitial edema, destruction of the alveolar capillary bed with red cells free in interstitial spaces, and a marked increase in the numbers of inflammatory cells in both the alveolar air spaces and the lung interstitium. This pattern of injury is uniform throughout both lungs. Baboons exposed to 100% O_2 for 96 hours while being simultaneously treated with aerosolized hrMnSOD showed a marked histologic protection. This is illustrated in Figure 11C. It shows an intact alveolar epithelial membrane with minimal interstitial edema and no significant inflammatory cells either in the airspaces or in the alveolar interstitial spaces. The lungs are not totally normal. There were patchy areas of injury and increased numbers of type II alveolar epithelial cells.

Endothelial cells demonstrate some swelling and vesiculation; however, the overall protecting was striking.

Thus, topical delivery of hrMnSOD provides marked protection of pulmonary oxygen toxicity in primates in addition to mice.

Table II

Inhibition of Pulmonary Oxygen Toxicity in Mice by Aerosolized hrMnSOD

Exposure Time (hr)	Treatment/ Dose(mg/ml)/ Duration (min.)	Crs (μl/cm H_2O)	D_{Lco} (μl/min/mmHg)	Lung Lavage		SOD Activity (U/ml)	
				Protein (μg/ml)	LDH (U/l)	Lavage	Serum
0	---	28.4 ± 0.5	13.6 ± 0.3	410 ± 119	58 ± 9	6 ± 1	13 ± 1
84	PBS / --- / 30	16.6 ± 2.0	11.5 ± 0.8	1666 ± 715	226 ± 42	6 ± 1	15 ± 1
84	MnSOD/0.1 / 60	15.5 ± 2.5	12.8 ± 0.5	1863 ± 642	260 ± 30	7 ± 1	16 ± 2
84	MnSOD/1.0 / 60	19.4 ± 1.6	13.6 ± 0.7	1135 ± 182	209 ± 25	13 ± 1*	35 ± 4*
84	MnSOD/10 / 30	25.2 ± 1.0*	15.3 ± 0.7*	379 ± 100	114 ± 12*	29 ± 6*	52 ± 7*

All values are mean ± S.E.M. ($N \geq 6$). Historical data used for naive (unexposed) results.

Treatment was by aerosol inhalation [mice placed a sealed chamber with the output of (aerosol generated by) a DeVilbiss Aerosonic ultrasonic nebulizer loaded with noted concentration of hrMnSOD or vehicle (phosphate buffered saline, PBS) passed through the chamber at 1 liter/minute for noted duration b.i.d. (every 12 hours)].

Abbreviations: Crs = respiratory system compliance; D_{Lco} = diffusion capacity of the lungs for carbon monoxide; LDH = lactate dehydrogenase.

*$p < 0.05$: Significant protection with MnSOD treatment versus control; Dunnett's t-test.

Table III

Inhibition of Pulmonary Oxygen Toxicity in Mice by Aerosolized hrMnSOD

Exposure Time (hr)	Treatment	Dose (mg/ml)	Crs (μl/cm H_2O)	Lung Lavage		
				Protein (μg/ml)	LDH (U/l)	Leukocytes ($x10^3$/ml)
0	----	----	22.1 ± 1.9	456 ± 160	71 ± 12	137 ± 9
84	PBS	----	14.8 ± 1.5	2733 ± 612	315 ± 40	158 ± 30
84	MnSOD	1	20.3 ± 1.8	1194 ± 272*	152 ± 42*	522 ± 133
84	MnSOD	10	20.5 ± 2.0*	1160 ± 80*	157 ±36*	824 ± 153

All values are mean ± S.E.M. ($N \geq 6$).

Treatment was by aerosol inhalation [mice placed a sealed chamber with the output of (aerosol generated by) a DeVilbiss Aerosonic ultrasonic nebulizer loaded with noted (dose) concentration of hrMnSOD or vehicle (phosphate buffered saline, PBS) passed through the chamber at 1 liter/minute for 30 minutes b.i.d. (every 12 hours)].

Abbreviations: Crs = respiratory system compliance; LDH = lactate dehydrogenase.

*$p < 0.05$: Significant protection with MnSOD treatment versus control; Dunnett's t-test.

Table IV

Inhibition of Pulmonary Oxygen Toxicity in Mice by Aerosolized hrMnSOD

Exposure Time (hr)	Treatment	Dose (mg/ml)	Crs (μl/cm H_2O)	D_{Lco} (μl/min/mmHg)	Lung Lavage		
					Protein (μg/ml)	LDH (U/l)	Leukocytes ($x10^3$/ml)
0	----	----	24.6 ± 1.2	19.0 ± 1.4	480 ± 90	51 ± 5	137 ± 9
84 (air)	PBS	----	24.0 ± 2.4	16.7 ± 3.8	518 ± 201	65 ± 13	187 ± 51
84 (O_2)	PBS	----	10.8 ± 1.6	13.1 ± 1.2	3094 ± 768	322 ± 25	169 ± 29
84 (O_2)	MnSOD	0.1	13.8 ± 1.9	15.0 ± 1.1	2147 ± 465	247 ± 50	310 ± 35*
84 (O_2)	MnSOD	1.0	20.5 ± 1.3*	17.0 ± 1.1*	397 ± 113*	143 ± 36*	327 ± 21*

All values are mean ± S.E.M. ($N \geq 6$).

Treatment was by aerosol inhalation [mice placed a sealed chamber with the output of (aerosol generated by) a DeVilbiss Aerosonic ultrasonic nebulizer loaded with noted (dose) concentration of hrMnSOD or vehicle (phosphate buffered saline, PBS) passed through the chamber at 1 liter/minute for 60 minutes once daily (every 24 hours)].

Abbreviations: Crs = respiratory system compliance; D_{Lco} = diffusion capacity of the lungs for carbon monoxide; LDH = lactate dehydrogenase.

*$p < 0.05$: Significant protection with MnSOD treatment versus control; Dunnett's t-test.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Boehringer Ingelheim Pharmaceuticals, Inc.

(ii) TITLE OF INVENTION: Method For Inhibiting Pulmonary Oxygen Toxicity Using Manganese Superoxide Dismutase

(iii) NUMBER OF SEQUENCES: 4

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Sterne, Kessler, Goldstein & Fox
(B) STREET: 1100 New York Avenue, Suite 600
(C) CITY: Washington
(D) STATE: D.C.
(E) COUNTRY: U.S.A.
(F) ZIP: 20005-3934

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US to be assigned
(B) FILING DATE: Herewith
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 08/036,604
(B) FILING DATE: 24-MAR-1993

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Esmond, Robert W.
(B) REGISTRATION NUMBER: 32,893
(C) REFERENCE/DOCKET NUMBER: 1011.0760001

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (202) 371-2600
(B) TELEFAX: (202) 371-2540

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 561 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATGAAGCACT CTTTGCCAGA CTTGCCATAC GACTACGGTG CTCTAGAACC ACACATCAAT 60

GCTCAAATCA TGCAATTGCA CCACTCTAAG CACCACGCGG CCTACGTGAA CAACCTGAAC 120

GTCACCGAGG AGAAGTACCA GCAGGCGTTG GCCAAGGGAG ATGTTACAGC CCAGATAGCT 180

CTTCAGCCTG CACTGAAGTT CAATGGTGGT GGTCATATCA ATCATAGCAT TTTCTGGACA 240

AACCTCAGCC CTAACGGTGG TGGAGAACCC AAAGGGGAGT TGCTGGAAGC CATCAAACGT 300

GACTTTGGTT CCTTTGACAA GTTTAAGGAG AAGCTGACGG CTGCATCTGT TGGTGTCCAA 360

GGCTCAGGTT GGGGTTGGCT TGTTTTCAAT CAGGATCCAC TGCAAGGAAC AACAGGCCTT 420

ATTCCACTGC TGGGGATTGA TGTGTGGGAG CACGCTTACT ACCTTCAGTA TAAAAATGTC 480

AGGCCTGATT ATCTAAAAGC TATTTGGAAT GTAATCAACT GGGAGAATGT AACTGAAAGA 540

TACATGGCTT GCAAAAAGTA A 561

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 561 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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GTCACCGAGG AGAAGTACCA GCAGGCGTTG GCCAAGGGAG ATGTTACAGC CCAGATAGCT 180

CTTCAGCCTG CACTGAAGTT CAATGGTGGT GGTCATATCA ATCATAGCAT TTTCTGGACA 240

AACCTCAGCC CTAACGGTGG TGGAGAACCC AAAGGGGAGT TGCTGGAAGC CATCAAACGT 300

GACTTTGGTT CCTTTGACAA GTTTAAGGAG AAGCTGACGG CTGCATCTGT TGGTGTCCAA 360

GGCTCAGGTT GGGGTTGGCT TGTTTTCAAT CAGGATCCAC TGCAAGGAAC AACAGGCCTT 420

ATTCCACTGC TGGGGATTGA TGTGTGGGAG CACGCTTACT ACCTTCAGTA TAAAAATGTC 480

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TACATGGCTT GCAAAAAGTA A 561

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 561 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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GTCACCGAGG AGAAGTACCA GCAGGCGTTG GCCAAGGGAG ATGTTACAGC CCAGATAGCT 180

CTTCAGCCTG CACTGAAGTT CAATGGTGGT GGTCATATCA ATCATAGCAT TTTCTGGACA 240

AACCTCAGCC CTAACGGTGG TGGAGAACCC AAAGGGGAGT TGCTGGAAGC CATCAAACGT 300

GACTTTGGTT CCTTTGACAA GTTTAAGGAG AAGCTGACGG CTGCATCTGT TGGTGTCCAA 360

GGCTCAGGTT GGGGTTGGCT TGTTTTCAAT CAGGATCCAC TGCAAGGAAC AACAGGCCTT 420

ATTCCACTGC TGGGGATTGA TGTGTGGGAG CACGCTTACT ACCTTCAGTA TAAAAATGTC 480

AGGCCTGATT ATCTAAAAGC TATTTGGAAT GTAATCAACT GGGAGAATGT AACTGAAAGA 540

TACATGGCTT GCAAAAAGTA A 561

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 561 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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GTCACCGAGG AGAAGTACCA GCAGGCGTTG GCCAAGGGAG ATGTTACAGC CCAGATAGCT 180

CTTCAGCCTG CACTGAAGTT CAATGGTGGT GGTCATATCA ATCATAGCAT TTTCTGGACA 240

AACCTCAGCC CTAACGGTGG TGGAGAACCC AAAGGGGAGT TGCTGGAAGC CATCAAACGT 300

GACTTTGGTT CCTTTGACAA GTTTAAGGAG AAGCTGACGG CTGCATCTGT TGGTGTCCAA 360

GGCTCAGGTT GGGGTTGGCT TGTTTTCAAT CAGGATCCAC TGCAAGGAAC AACAGGCCTT 420

ATTCCACTGC TGGGGATTGA TGTGTGGGAG CACGCTTACT ACCTTCAGTA TAAAAATGTC 480

AGGCCTGATT ATCTAAAAGC TATTTGGAAT GTAATCAACT GGGAGAATGT AACTGAAAGA 540

TACATGGCTT GCAAAAAGTA A 561

What Is Claimed Is:

1. A method for inhibiting pulmonary oxygen toxicity in a patient susceptible thereto, which comprises topically administering to the patient, an effective amount of a human MnSOD tetramer.

2. A method as recited in claim 1 wherein the topical administration is by intranasal insufflation.

3. A method as recited in claim 1 wherein the topical administration is by inhalation.

4. A method as recited in claim 1 wherein the topical administration is by intratracheal instillation.

5. A method as recited in claim 1 wherein the topical administration is prophylactic.

6. A method as recited in claim 1 wherein the human MnSOD tetramer is encoded by the DNA sequence

5' ATG AAG CAC TCT TTG CCA GAC TTG CCA TAC GAC TAC GGT
GCT CTA GAA CCA CAC ATC AAT GCT CAA ATC ATG CAA TTG
CAC CAC TCT AAG CAC CAC GCG GCC TAC GTG AAC AAC CTG
AAC GTC ACC GAG GAG AAG TAC CAG CAG GCG TTG GCC AAG
GGA GAT GTT ACA GCC CAG ATA GCT CTT CAG CCT GCA CTG
AAG TTC AAT GGT GGT GGT CAT ATC AAT CAT AGC ATT TTC
TGG ACA AAC CTC AGC CCT AAC GGT GGT GGA GAA CCC AAA
GGG GAG TTG CTG GAA GCC ATC AAA CGT GAC TTT GGT TCC
TTT GAC AAG TTT AAG GAG AAG CTG ACG GCT GCA TCT GTT
GGT GTC CAA GGC TCA GGT TGG GGT TGG CTT GTT TTC AAT
CAG GAT CCA CTG CAA GGA ACA ACA GGC CTT ATT CCA CTG
CTG GGG ATT GAT GTG TGG GAG CAC GCT TAC TAC CTT CAG
TAT AAA AAT GTC AGG CCT GAT TAT CTA AAA GCT ATT TGG
AAT GTA ATC AAC TGG GAG AAT GTA ACT GAA AGA TAC ATG
GCT TGC AAA AAG TAA [SEQ ID NO:1]

wherein AAG at codon 30 can be replaced by CAG and CAC at codon 32 can be replaced by CAT [SEQ ID NO:2], [SEQ ID NO:3] and [SEQ ID NO:4].

7. A method as recited in claim 4 wherein the human MnSOD tetramer is prepared by transforming a eukaryotic host with a vector comprising the sequence

```
5'   ATG AAG CAC TCT TTG CCA GAC TTG CCA TAC GAC TAC GGT
     GCT CTA GAA CCA CAC ATC AAT GCT CAA ATC ATG CAA TTG
     CAC CAC TCT AAG CAC CAC GCG GCC TAC GTG AAC AAC CTG
     AAC GTC ACC GAG GAG AAG TAC CAG CAG GCG TTG GCC AAG
     GGA GAT GTT ACA GCC CAG ATA GCT CTT CAG CCT GCA CTG
     AAG TTC AAT GGT GGT GGT CAT ATC AAT CAT AGC ATT TTC
     TGG ACA AAC CTC AGC CCT AAC GGT GGT GGA GAA CCC AAA
     GGG GAG TTG CTG GAA GCC ATC AAA CGT GAC TTT GGT TCC
     TTT GAC AAG TTT AAG GAG AAG CTG ACG GCT GCA TCT GTT
     GGT GTC CAA GGC TCA GGT TGG GGT TGG CTT GTT TTC AAT
     CAG GAT CCA CTG CAA GGA ACA ACA GGC CTT ATT CCA CTG
     CTG GGG ATT GAT GTG TGG GAG CAC GCT TAC TAC CTT CAG
     TAT AAA AAT GTC AGG CCT GAT TAT CTA AAA GCT ATT TGG
     AAT GTA ATC AAC TGG GAG AAT GTA ACT GAA AGA TAC ATG
     GCT TGC AAA AAG TAA      [SEQ ID NO:1]
```

wherein AAG at codon 30 can be replaced by CAG and CAC at codon 32 can be replaced by CAT [SEQ ID NO:2], [SEQ ID NO:3] and [SEQ ID NO:4].

8. A method as recited in claim 7 wherein the eukaryotic host is yeast.

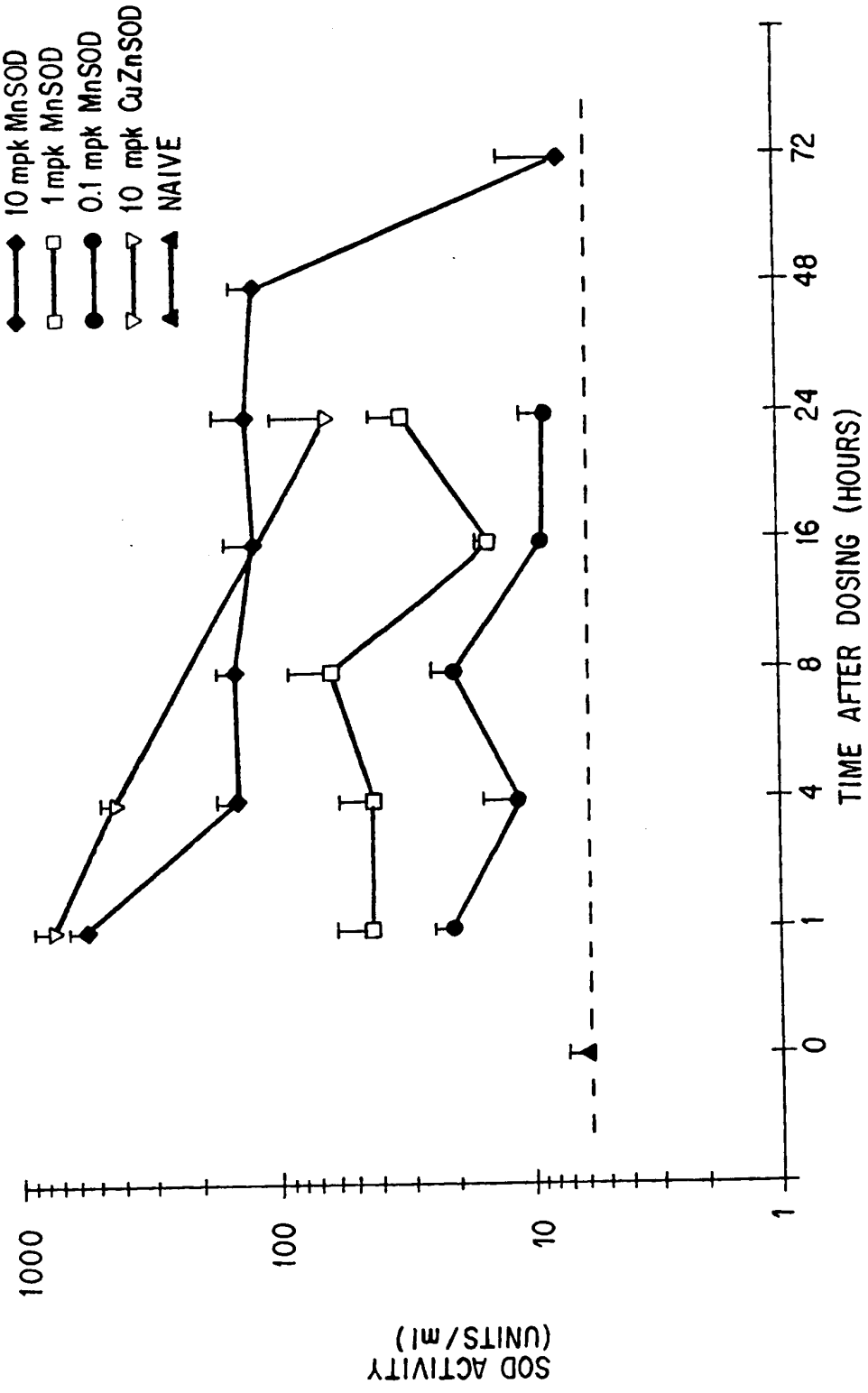

10 mpk MnSOD
1 mpk MnSOD
0.1 mpk MnSOD
10 mpk CuZnSOD
NAIVE
1000
100
10
1
SOD ACTIVITY
(UNITS/ml)
0
1
4
8
16
24
48
72
TIME AFTER DOSING (HOURS)

FIG. 1A

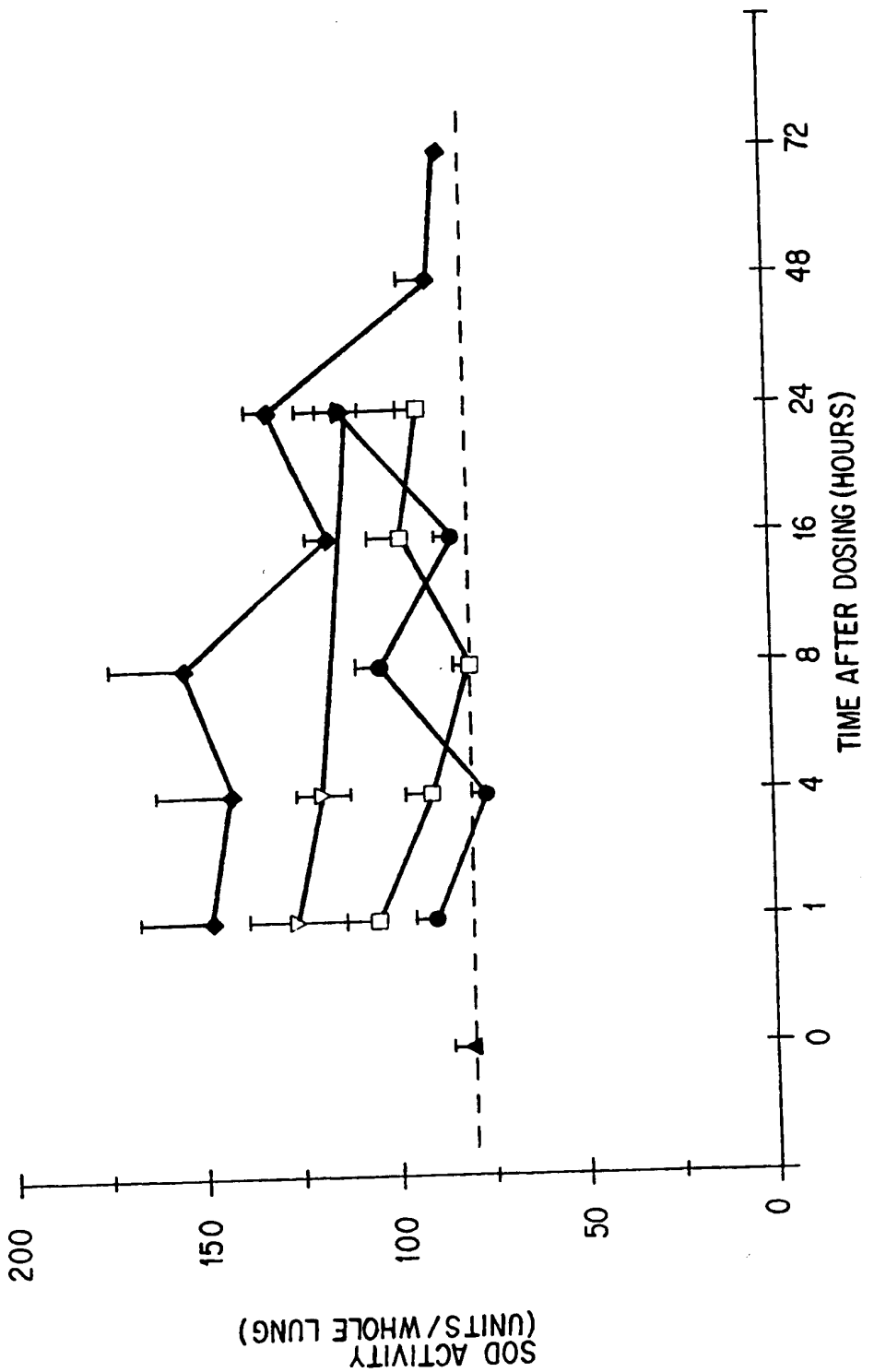

SOD ACTIVITY
(UNITS/WHOLE LUNG)
200
150
100
50
0
0
1
4
8
16
24
48
72
TIME AFTER DOSING (HOURS)

FIG. 1B

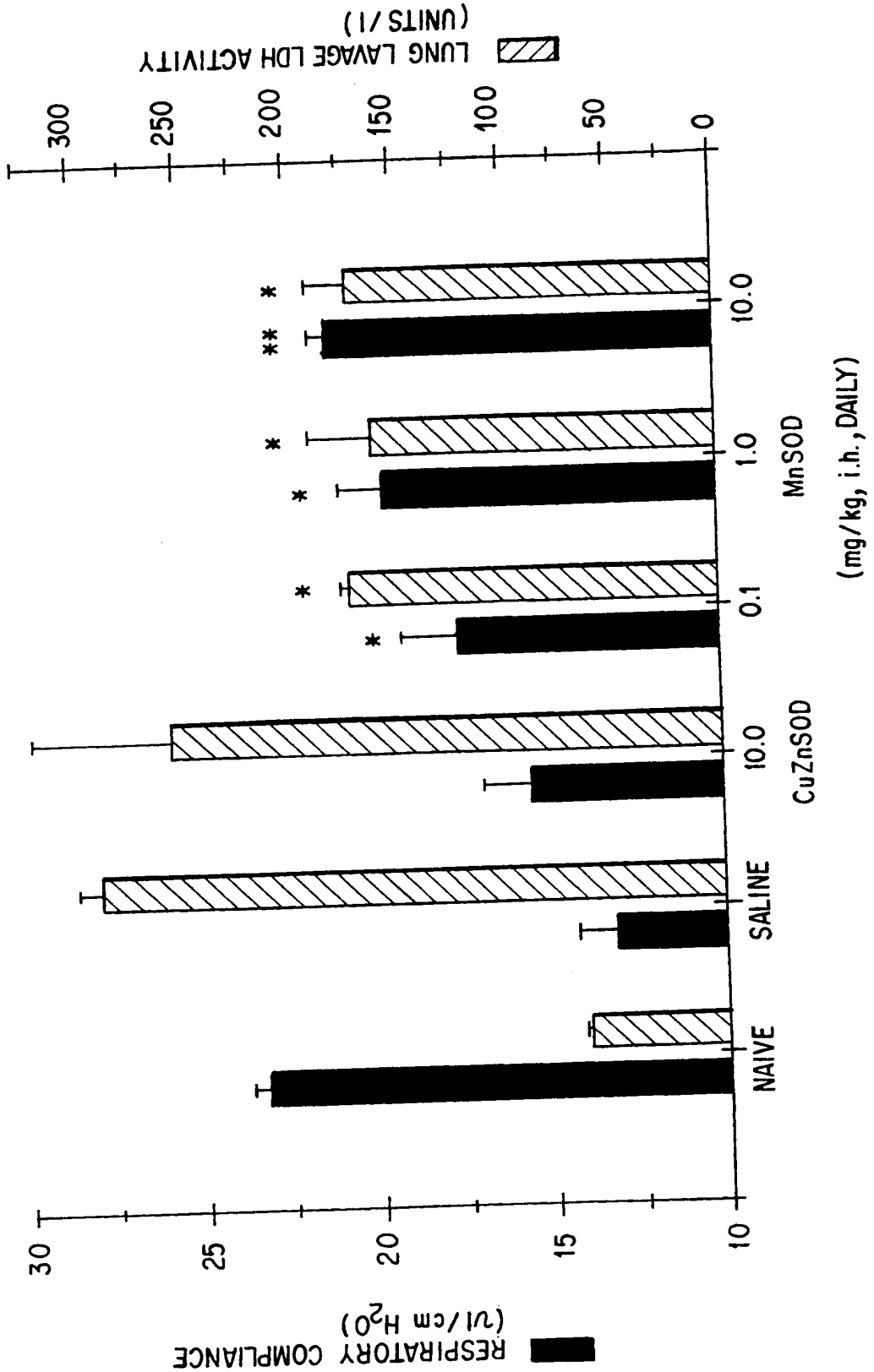

LUNG LAVAGE LDH ACTIVITY
(UNITS/l)
300
250
200
150
100
50
0
10.0
1.0
0.1
10.0
SALINE
NAIVE
MnSOD
CuZnSOD
(mg/kg, i.h., DAILY)
RESPIRATORY COMPLIANCE
(ml/cm H2O)
30
25
20
15
10

FIG. 2

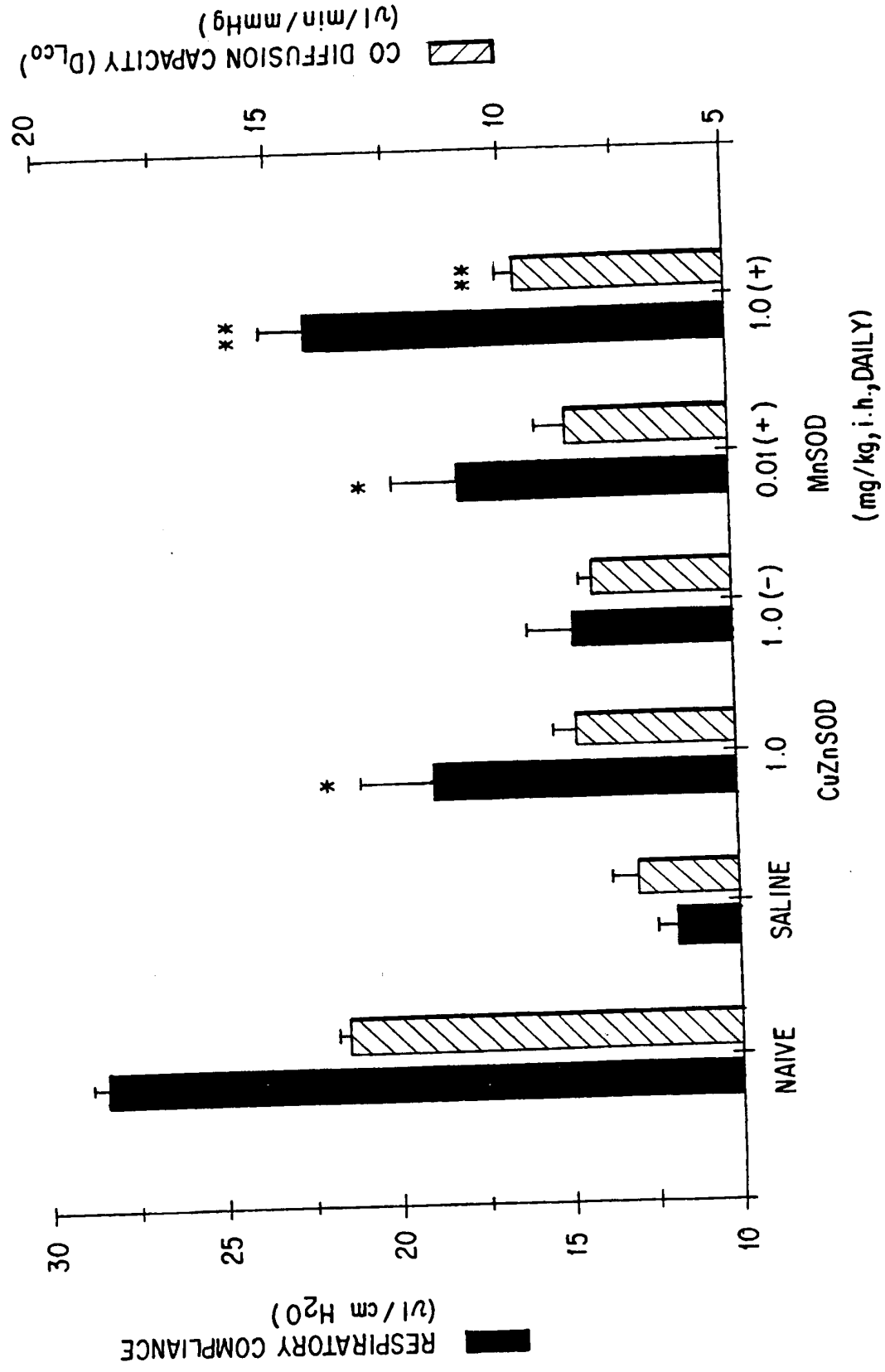

CO DIFFUSION CAPACITY (D_{LCO})
(μl/min/mmHg)
20
15
10
5
**
**
*
*
1.0(+)
0.01(+)
1.0(-)
1.0
SALINE
NAIVE
MnSOD
CuZnSOD
(mg/kg, i.h., DAILY)
RESPIRATORY COMPLIANCE
(μl/cm H_2O)
30
25
20
15
10

FIG.3A

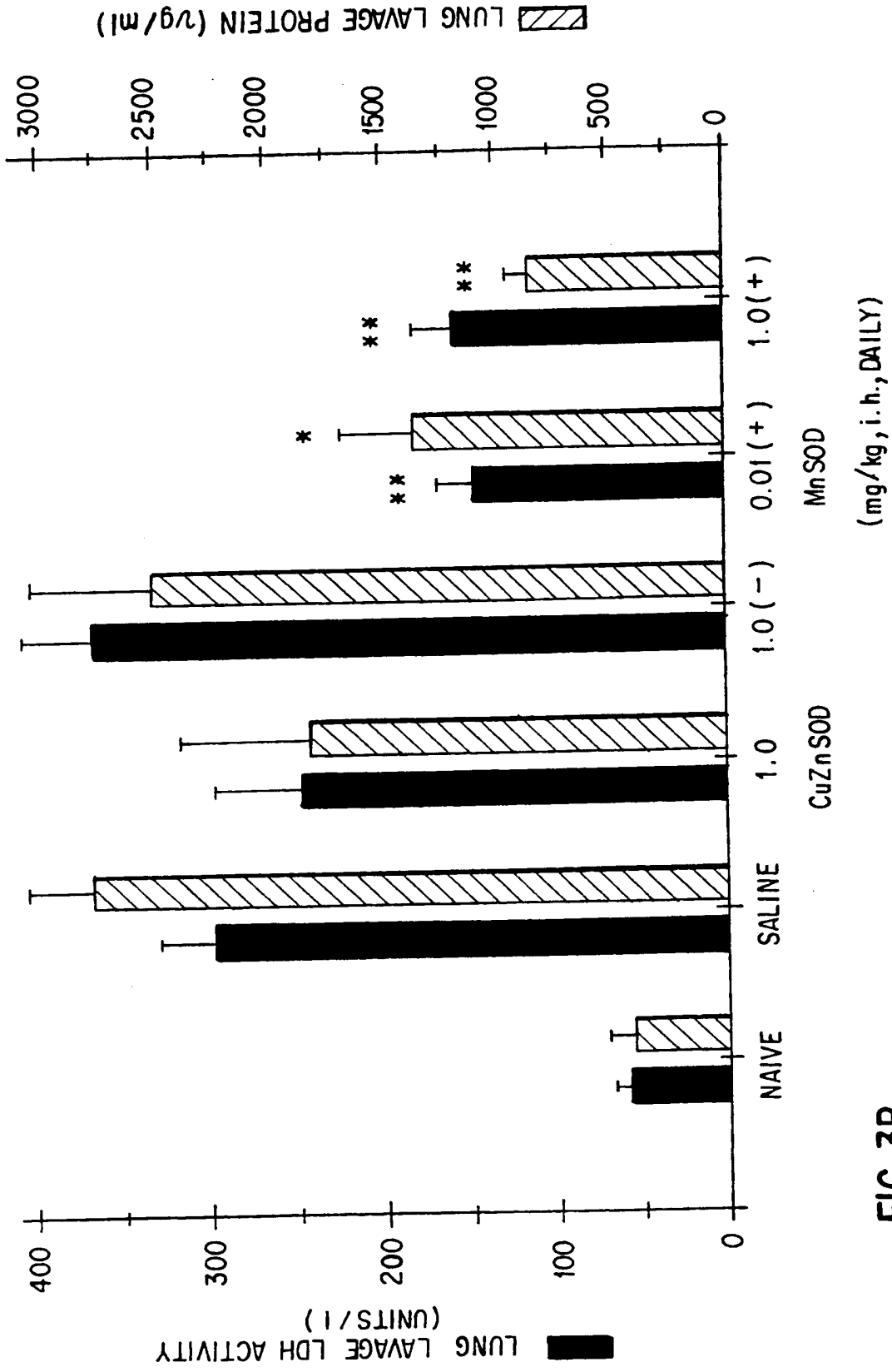

LUNG LAVAGE PROTEIN (μg/ml)
3000
2500
2000
1500
1000
500
0
**
**
*
**
NAIVE
SALINE
CuZnSOD
1.0
1.0(−)
MnSOD
0.01(+)
1.0(+)
(mg/kg, i.h., DAILY)
400
300
200
100
0
LUNG LAVAGE LDH ACTIVITY
(UNITS/l)

FIG. 3B

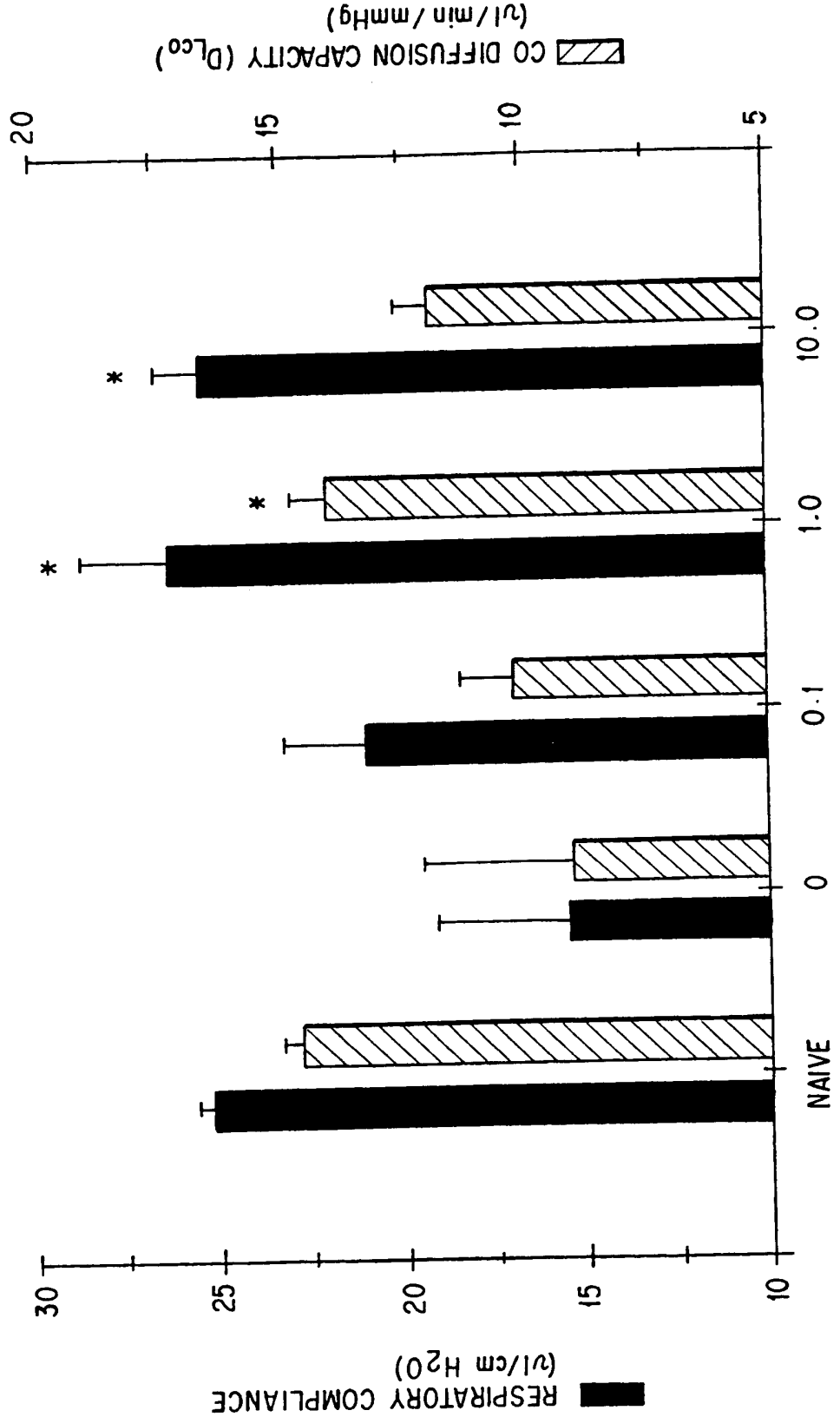

CO DIFFUSION CAPACITY (D_{LCO})
(ul/min/mmHg)
20
15
10
5
10.0
1.0
0.1
0
NAIVE
EXPOSURE + MnSOD TREATED
(mg/kg, i.h., DAILY)
*
*
*
RESPIRATORY COMPLIANCE
(ul/cm H_2O)
30
25
20
15
10

FIG.4A

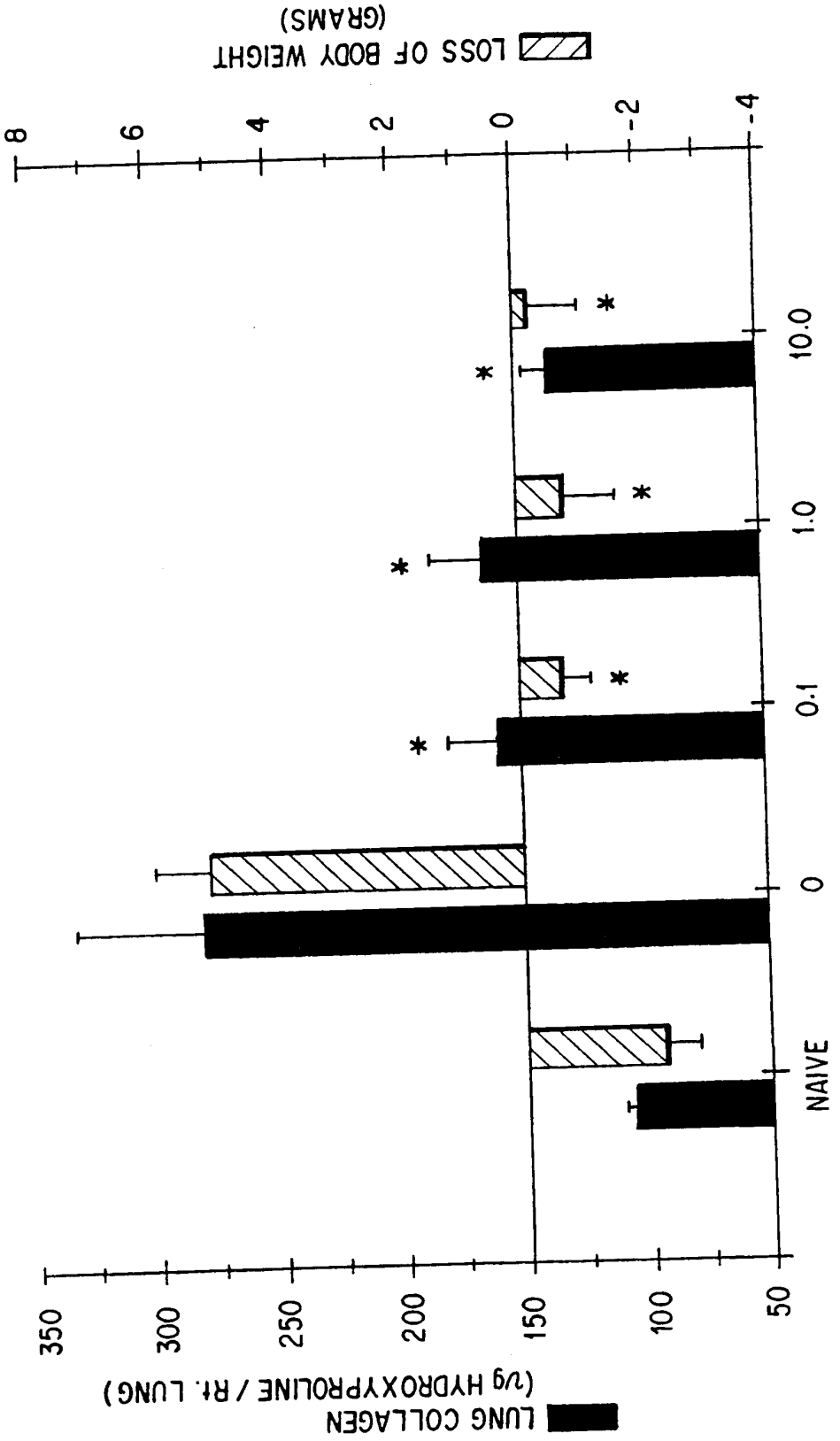

LOSS OF BODY WEIGHT
(GRAMS)
8
6
4
2
0
-2
-4
*
10.0
1.0
0.1
0
NAIVE
EXPOSURE+MnSOD TREATED
(mg/kg, i.h., DAILY)
350
300
250
200
150
100
50
LUNG COLLAGEN
(μg HYDROXYPROLINE / Rt. LUNG)

FIG. 4B

FIG. 5D

FIG. 5H

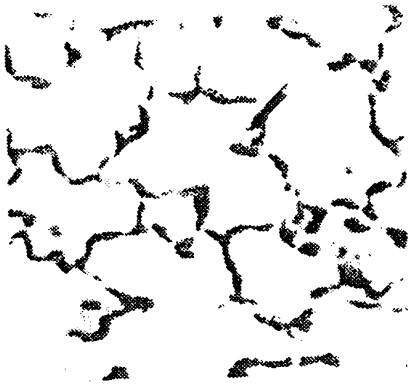


FIG. 5C

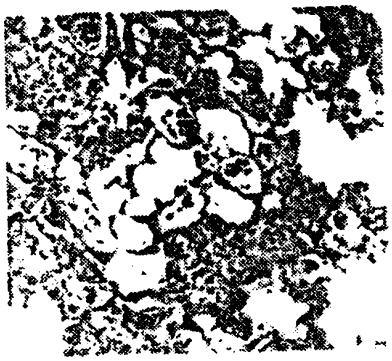


FIG. 5G

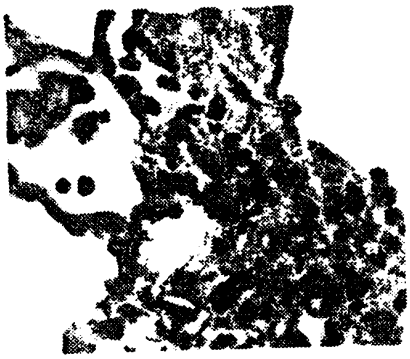


FIG. 5B

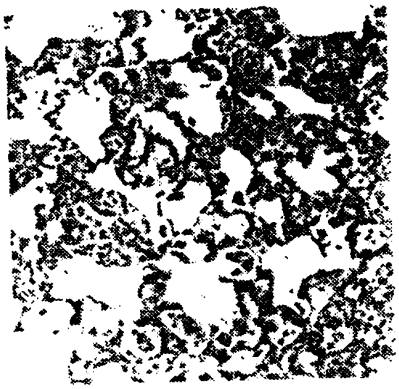


FIG. 5F

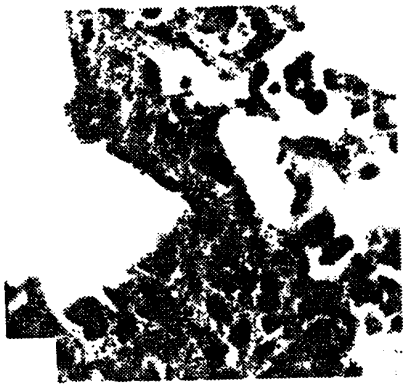
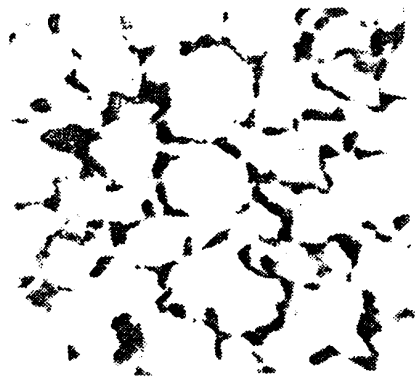


FIG. 5A

FIG. 5E



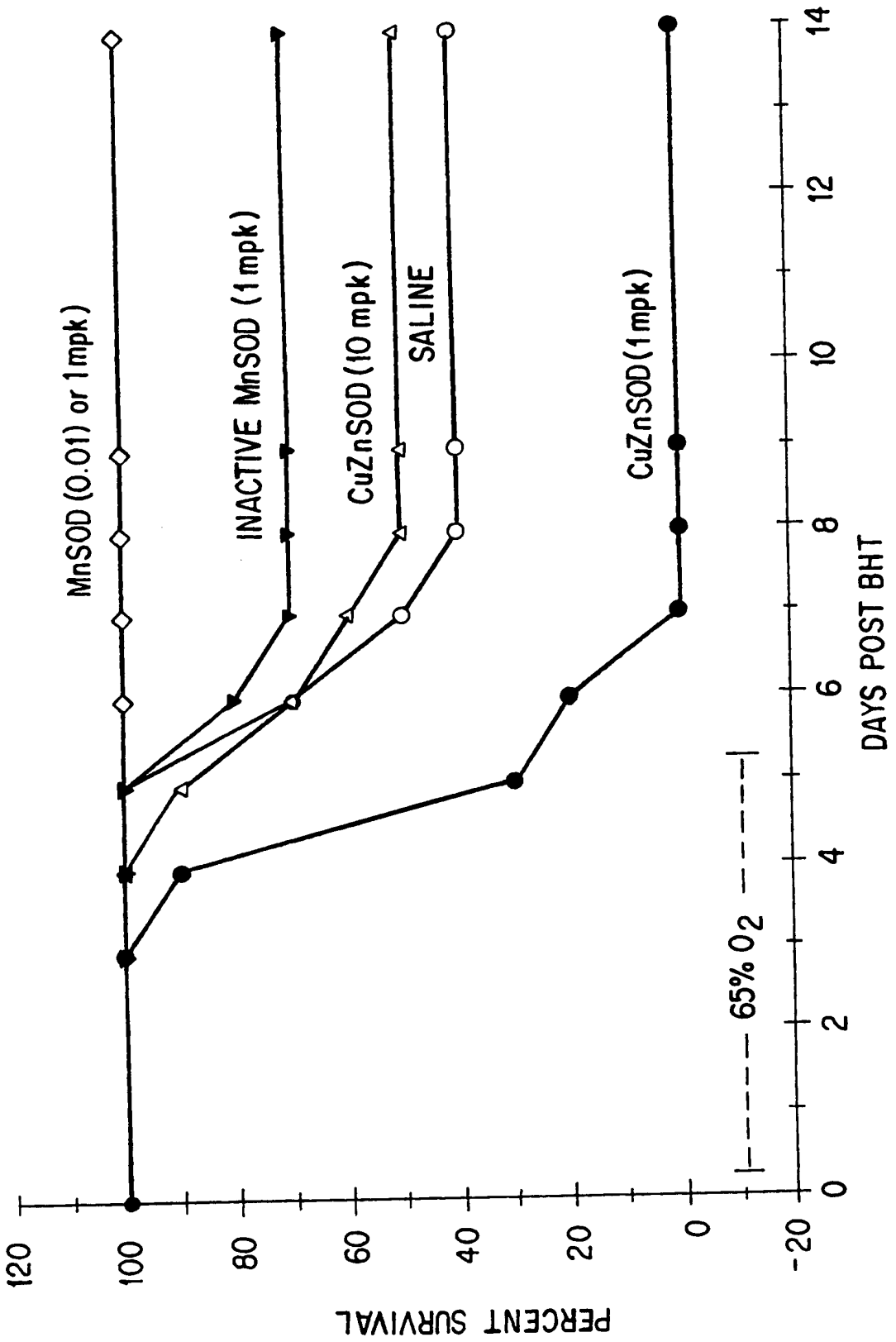

MnSOD (0.01) or 1mpk)
INACTIVE MnSOD (1mpk)
CuZnSOD (10 mpk)
SALINE
CuZnSOD (1mpk)
65% O2
DAYS POST BHT
PERCENT SURVIVAL
120
100
80
60
40
20
0
-20
0
2
4
6
8
10
12
14

FIG.6

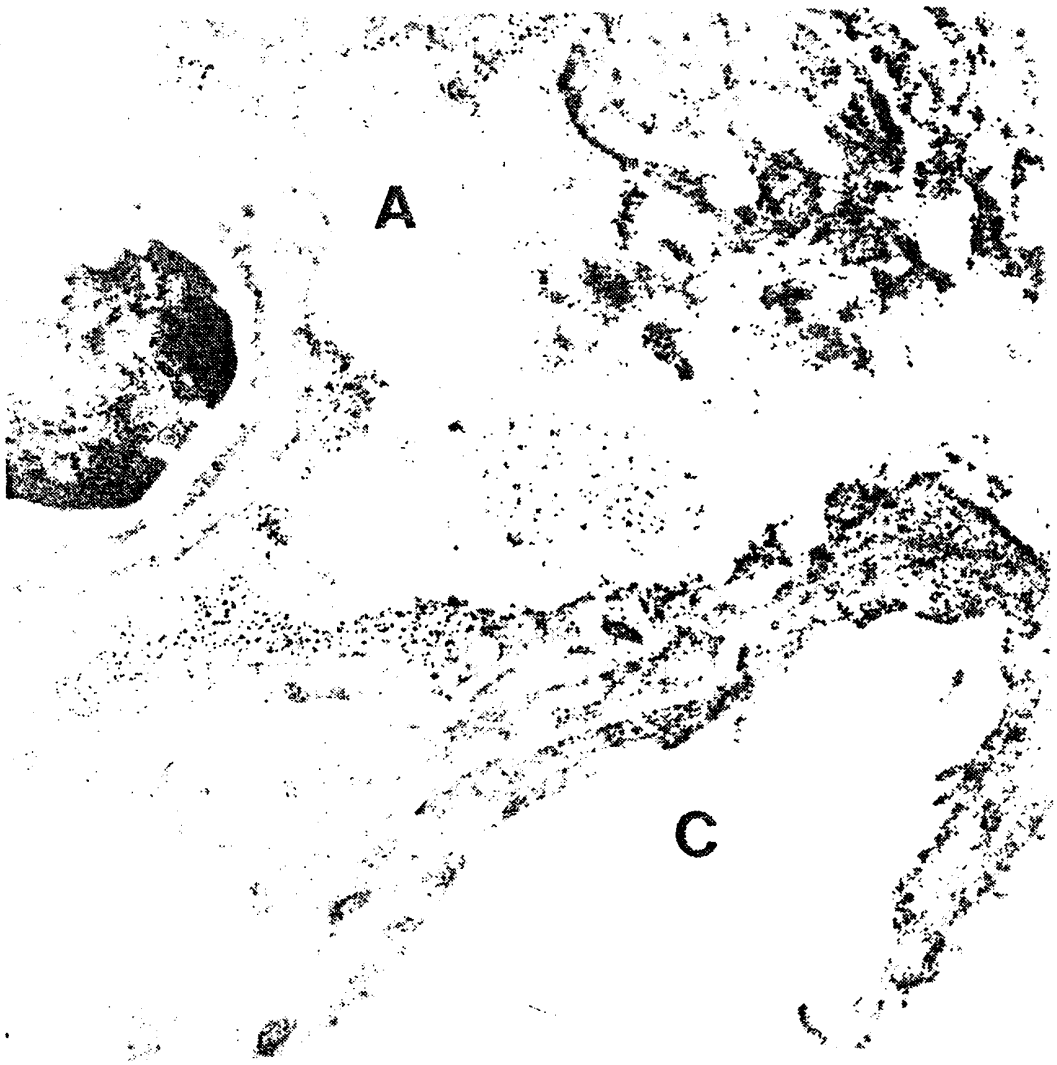


FIG. 7

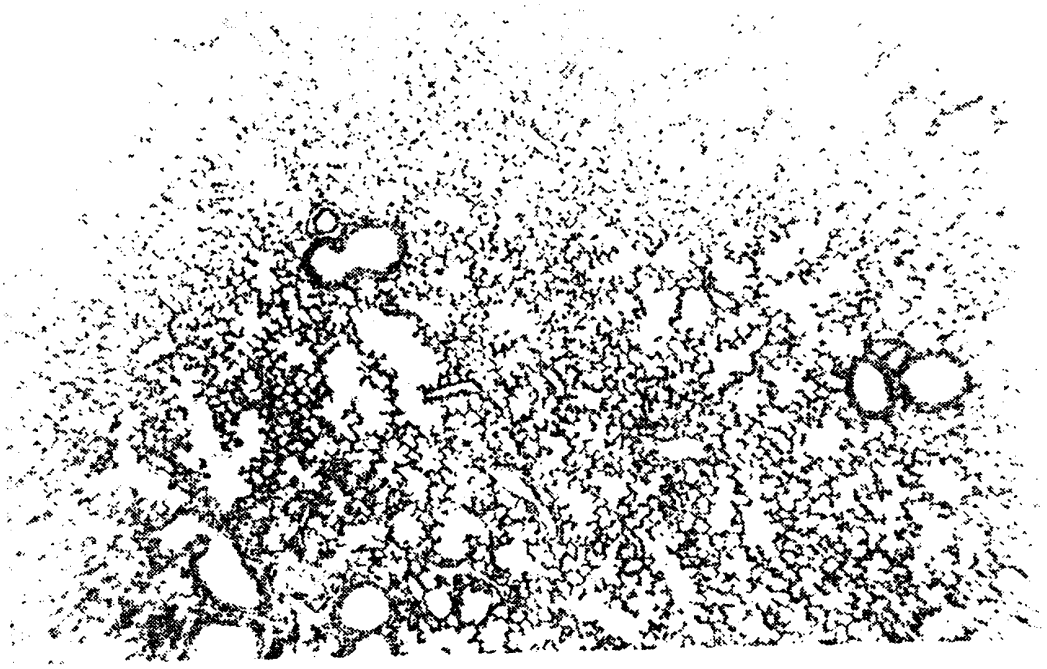


FIG. 8A

FIG. 8B

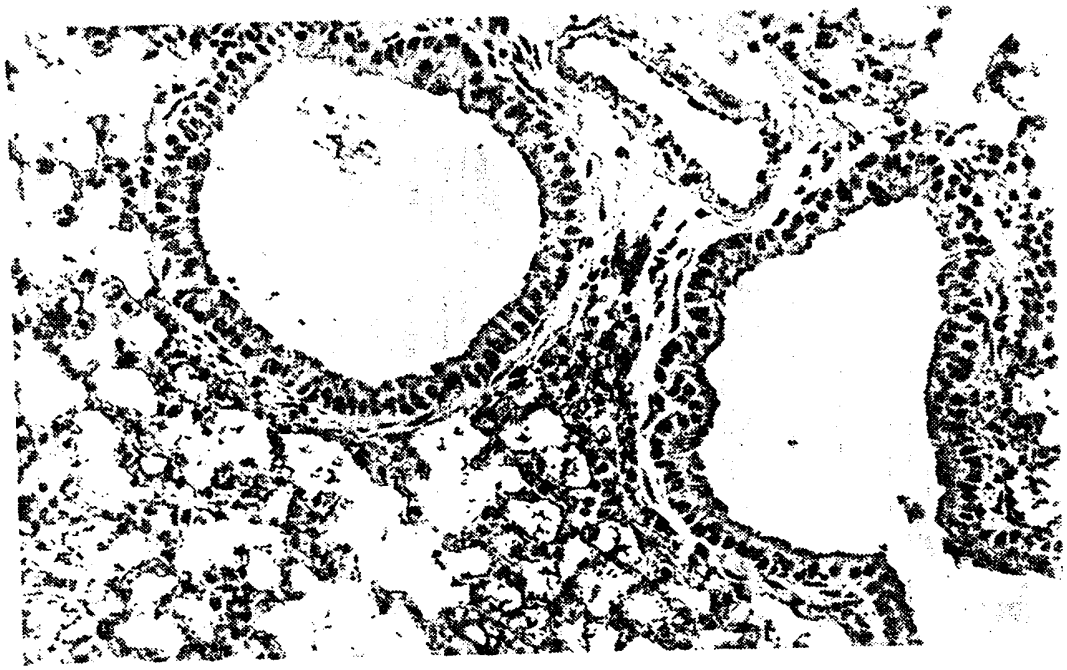


FIG. 8C

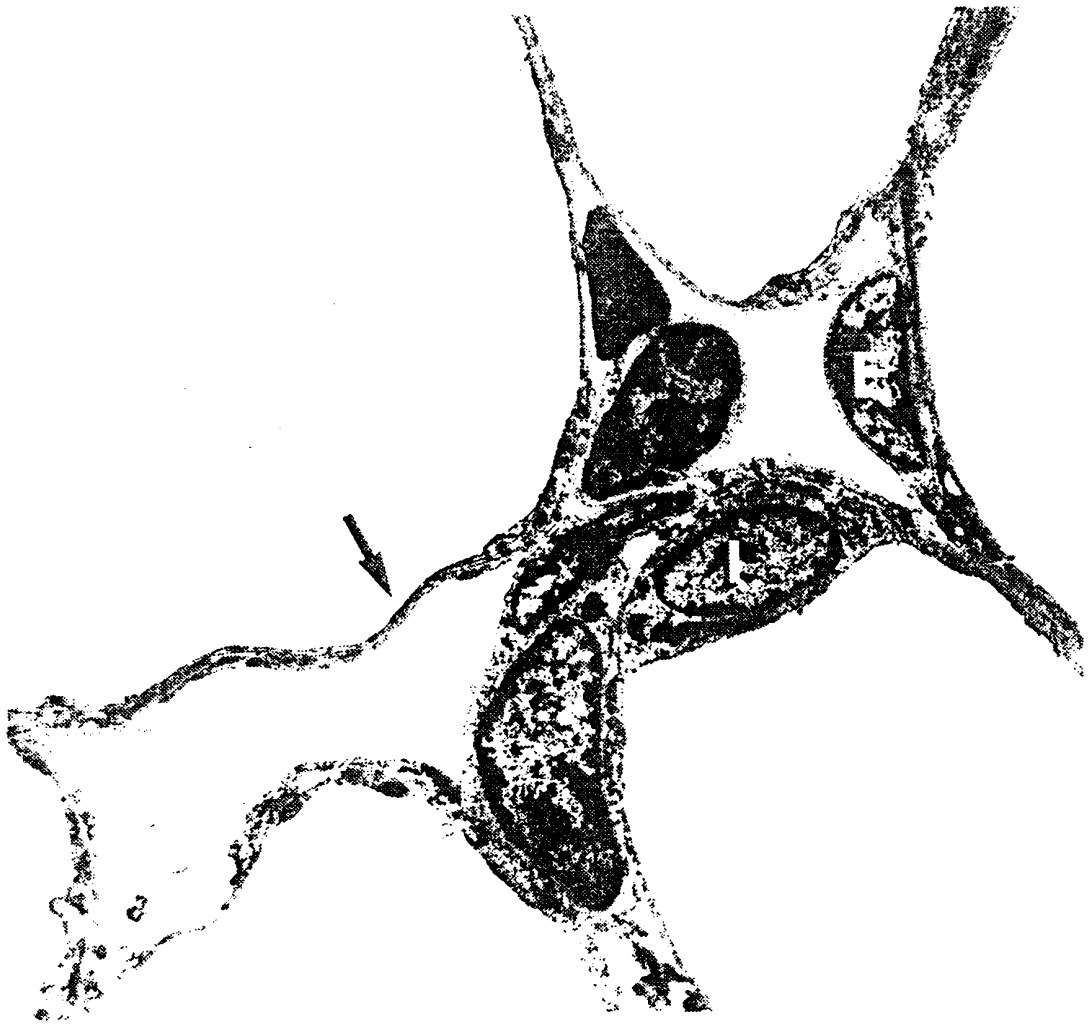


FIG. 9A

FIG. 9B

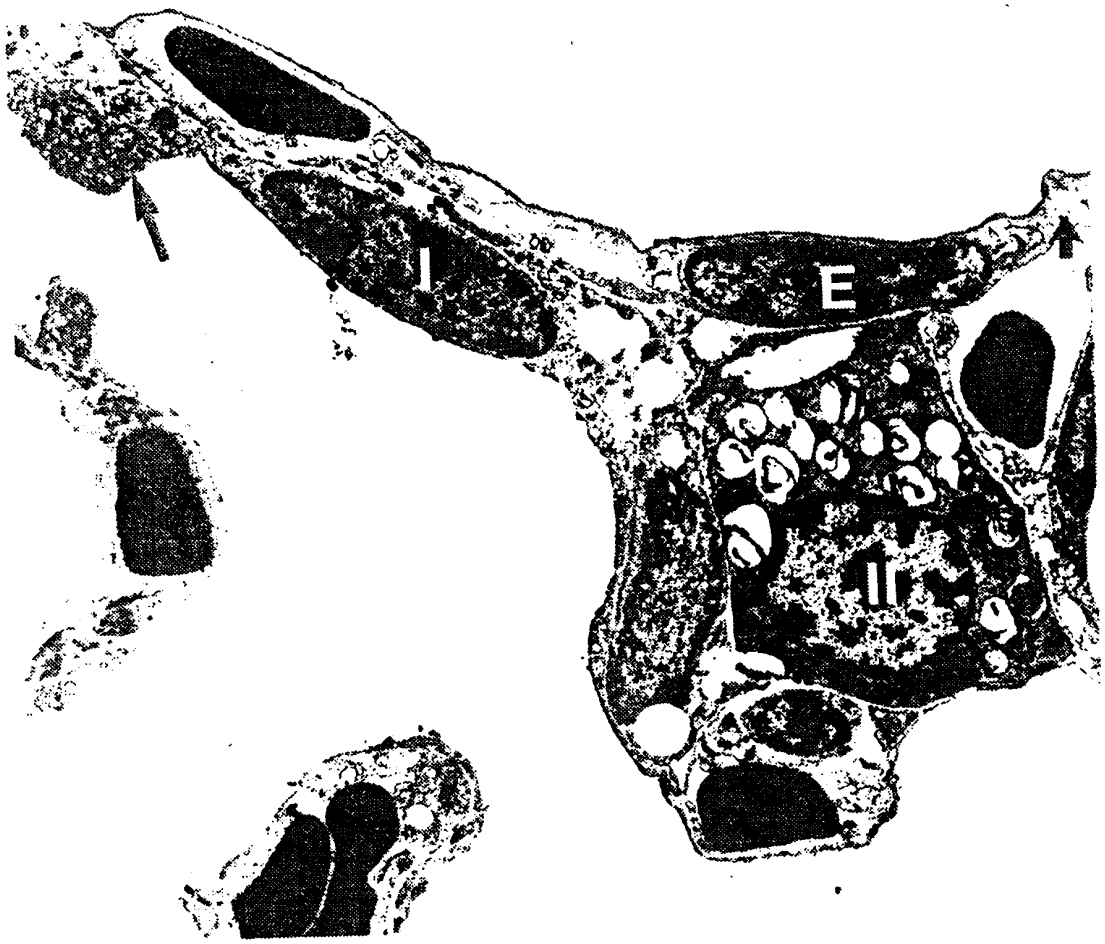


FIG. 9C

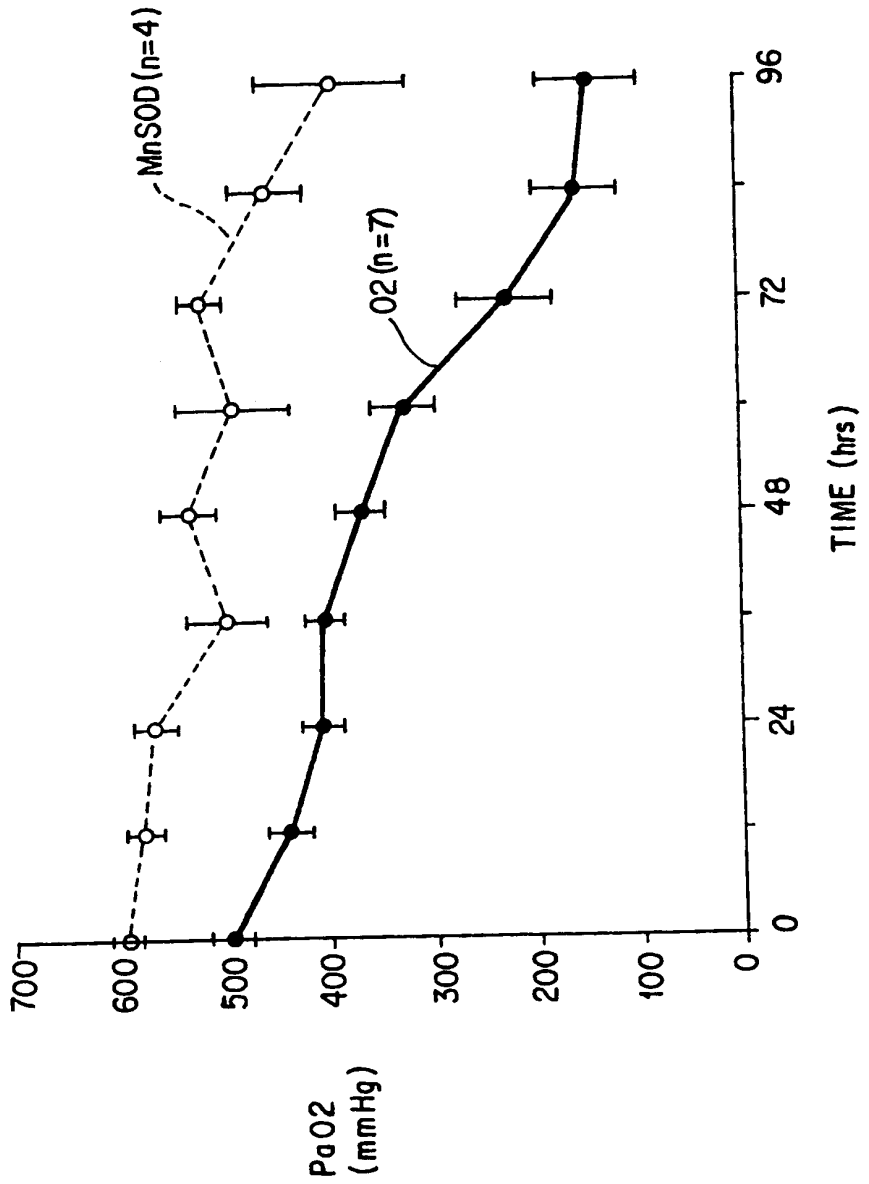

MnSOD(n=4)
O2(n=7)
700
600
500
400
300
200
100
0
PaO2
(mmHg)
0
24
48
72
96
TIME (hrs)

FIG. 10

FIG. 11A

FIG. 11B

FIG. 11C

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/03185

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 37/50; C07K 13/00; C12N 15/53, 15/81
US CL :424/94.4; 435/69.9, 189
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/94.4; 435/69.9, 189

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, BIOSIS, WORLD PATENTS INDEX

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 5,116,616 (GONENNE) 26 May 1992, see entire document.	1-8
Y	EP, A, 0,282,899 (HECKL ET AL) 21 September 1988, see entire document.	1-8
Y	EP, A, 0,284,105 (HARTMAN ET AL) 28 SEPTEMBER 1988, see entire document.	1-8
A	European Journal of Biochemistry, Volume 194, issued 1990, Y. Matuda et al, "Human Liver Manganese Superoxide Dismutase: Purification and Crystallization, Subunit Association and Sulfhydryl Reactivity", pages 713-720, see page 713.	1-8

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
26 APRIL 1994

Date of mailing of the international search report
MAY 16 1994

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